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13. ABSTRACT *(Maximum 200 words)*

We are interested in studying the role of integrins in the regulation of cell proliferation in normal and transformed mammary epithelial cells. During this past year, we have observed a possible difference in the abilities of integrins to stimulate cell prolieration in normal mammary epithelial cells but not in the highly metastatic carcinoma cell line MDA-MB-435. Differences in the strength of this stimulation among integrins may represent integrin-specific effects or possibly the level of cell surface expression. The mechanisms for stimulation of cell cycle progression through G1 by integrins remain unclear. To date, we have not observed significant impacts of integrin clustering on the time course of cyclin Ds expression, Rb phosphorylation, nor the association of growth factor receptors with the integrin complexes. Significant differences between normal and tumor mammary epithelial cells were observed for the expression of cyclin D2 and in the isoforms of the integrin a6, a6A, and a6B. During this next year, we hope to clarify the reason(s) for the inability of integrins to stimulate cell proliferation of MDA-MB-435 cells and the significances of the loss of cyclin D2 expression and changes in integrin a6 isoform expression.

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I. INTRODUCTION

A. Nature of the Problem

Breast cancer is among the leading causes of cancer-related death among women in this country and the incidence of breast cancer among young women is rising. Early detection offers the greatest hope for survival and, in recent years, the mortality rates have started to decline. The past few years have also seen several discoveries that may lead to the development of novel therapeutics in the near future. Continued development of new strategies to improve breast cancer survival will depend upon continued study of the pathogenesis of breast cancer and the events which lead to neoplastic transformation of breast epithelial cells.

Cancer cells differ from normal cells in that they undergo uncontrolled cell growth and acquire the abilities to invade adjacent tissues, enter the circulatory and lymphatic systems, and "home" to sites distal from the primary tumor (metastasis). An important aspect of tumor invasion and metastasis involves the adhesive interactions of tumor cells with other cells and the extracellular matrix. The integrin family of cell adhesion receptors (reviewed by (16)) mediates many of these adhesive interactions. Integrins provide not only a structural means of cell anchorage but also a means of transmitting signals regulating gene expression and protein function (8). Several groups have reported a role of integrins in regulating the progression of cells through the cell cycle (25, 27, 32). It is unclear if significant differences exist between normal and transformed cells. **Our efforts are focused to understand how integrins participate in the regulation of cell division in normal breast cells and to determine how breast cancer cells escape these regulatory pathways.**

B. Background of Previous Work

1. Integrins. Integrins are transmembrane glycoproteins comprised of two non-covalently associated subunits (α and β) that mediate both cell-cell and cell-substrate adhesion. Integrin receptors bind extracellular matrix (ECM) and plasma proteins, non-integrin adhesion receptors and other integrins (16). In addition to their role as primary mediators of cell adhesion, it is now become clear that integrins are also capable of transducing signals to the cell interior (8, 12, 16, 18, 19). Tyrosine phosphorylation appears to be a key aspect of integrin mediated signal transduction and a tyrosine kinase (p125 FAK) has been identified which localizes to focal adhesions when cells adhere to an ECM protein-coated surface (30). In addition, evidence is accumulating which suggests that integrin-mediated signaling events can induce gene expression (42) and affect transit through the cell cycle (25, 27, 32). Many of the ECM proteins that serve as ligands for integrins have been identified and include: fibronectin (FN), vitronectin (VN), laminins, and collagens. Some integrins can interact with more than one ligand (e.g. $\alpha 4\beta 1$, $\alpha v\beta 3$, $\alpha 3\beta 1$) while several ECM proteins are recognized by more than one integrin. For example, FN has been shown to interact with multiple integrin receptors ($\alpha 5\beta 1$, $\alpha 4\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$, $\alpha v\beta 1$, $\alpha 3\beta 1$)

2. Cell cycle Regulation. The initiation of cell division and transitions between different stages of the cell cycle involves signals that activate the association of specific protein complexes (cell cycle dependent kinases (cdk) with the regulatory cyclins). At each checkpoint certain cyclins and CDKs form active complexes which phosphorylate and thereby activate specific proteins necessary for DNA replication (G1/S), mitosis, and cytokinesis (G2/M). Recently, it has been shown that the activity of cyclin/CDK complexes is regulated by a family of CDK inhibitor proteins or CDIs (p16, p21, p27) that bind to and inactivate the CDKs (15).

The original purpose of this project was to determine how signals transduced via integrin receptors, especially a5b1, regulated the formation of cdk/cyclin complexes in neoplastic and normal breast cells. This tenet was based on observations that suggested that binding of the fibronectin peptide ligand, GRGDS, to the integrin $\alpha 5\beta 1$ regulated cell proliferation through modulation of cdc2/cyclinA complexes in partially transformed but not fully transformed epithelial cells (33). However, when we screened a number of mammary carcinoma cell lines, we did not observe a consistent effect GRGDS peptides on either cdc2 kinase activity or cyclinA associated kinase activities which correlated with either the relative degree of transformation or the pattern of expression of RGD-binding integrins. In addition, immunohistochemical analyses suggested that the integrin a5b1was not expressed by either normal mammary epithelial cells or on tumor cells. The stimulation of cdc2 kinase and cyclin A associated kinase activity by RGD peptides along with the stimulation of Rb phosphorylation by integrin clustering in synchronized cultures of normal human mammary epithelial cells suggests that multiple integrins are capable of regulating the cell cycle machinery.

Characterization of normal human mammary epithelial cells (HMEC) either isolated directly from breast reduction tissue or obtained commercially from Clonetics (San Diego, CA) and the mammary epithelial cell line MCF12A suggested that HMEC cultures resembled basal mammary epithelial cells while MCF12A cultures resembled luminal mammary epithelial cells. Thus, the MCF12A cell line may serve as an appropriate model for studying growth regulation of normal luminal epithelial cells, which is the source of most breast carcinomas.

3. The Statement of Work and Specific Aims are:

Statement of Work:
The purpose of this project is to determine the roles of integrins in regulating the proliferation of normal and neoplastic mammary epithelial cells.

Specific Aims:
Aim 1: Determine whether all integrins or a subset of integrins are involved in regulating normal mammary epithelial cell proliferation.

Aim 2: Determine the mechanisms of integrin-mediated cell cycle regulation.

Aim 3. Examine carcinoma cells for possible defects in these mechanisms.

C. Purpose of the Present Work

Most of the work for the past year has been to expand our studies to include the MCF12A and MDA-MB-435 cell lines. We have also attempted to identify possible mechanisms of integrin regulation of the cell cycle by looking at effects on RB phosphorylation and the expression of cell cycle cyclins (cyclin D1, D2, D3 and cyclin A, primarily).

II. BODY

A. Methods

1. Patient Samples

Tissue specimens were obtained from lumpectomy and mastectomy tissues provided by Dr. Ron Tickman who is a staff pathologist in the Laboratory of Pathology, Swedish Hospital Medical Center, Seattle, WA and used for immunohistochemistry and the isolation of breast cells (BC). These samples were removed from patients undergoing surgery at Swedish Hospital solely for diagnostic or therapeutic purposes and would otherwise be discarded. Samples were coded to permit access to information concerning the patient's age and sex, tumor histology, estrogen and progesterone receptor status, and the presence and location of metastases. This will enable me to correlate tumor type, malignancy, integrin expression and response to integrin ligation. Although no patient samples will be excluded on the basis of race, age, sex, religion, or ethnic background, the low incidence of male breast cancer will preclude collection of a large number of samples from men. I have received IRB approval from both Swedish hospital and SBRI for the use of these tissues (See Appendix).

2. Monoclonal and Polyclonal Antibodies

Anti-integrin Monoclonal Antibodies. Several monoclonal antibodies (Mabs) directed to a variety of integrin receptors expressed by normal and neoplastic epithelial cells have been produced, previously (6, 7, 38-40). Many of these Mabs perturb integrin function and will be used to determine how normal and neoplastic breast cells interact with FN or GRGDS peptide. These Mabs include the two used by Dr. Symington to ligate $\alpha 5$ or $\beta 1$ (P1D6 (anti- $\alpha 5$) and P4C10 (anti- $\beta 1$)) as well as inhibitory antibodies P1H5 (anti-$\alpha 2$), P1B5 ($\alpha 3$), P4C2 ($\alpha 4$), P5H9 ($\alpha v \beta 5$), LM609 ($\alpha v \beta 3$, courtesy of Dr. David Cheresh, The Scripps Research Institute, La Jolla, CA), and GoH3 (anti-$\alpha 6$, Pharmingen, San Diego, CA). Other anti-integrin antibodies include AA3 (anti-$\beta 4$, courtesy of Dr. Vito Quaranta, The Scripps Research Institute, La Jolla, CA), 3E1 (anti-$\beta 4$, Chemicon Inc., Temecula, CA), and L230 (anti-αV, American Type Culture Collection, Rockville, MD).

Antibodies Directed to Human Cell Cycle Proteins. Monoclonal or polyclonal antibodies directed to human CDKs (cdc2, cdk2-cdk5), cyclins (A, B1, D1, D2, D3, E), CDIs (p16, p21, p27), and tumor suppressor proteins (Rb and p53) were obtained from Pharmingen (San Diego, CA).

3. Breast Cell Isolation and Culture

Normal human mammary epithelial cells (HMEC) were obtained from Clonetics Corp (San Diego, CA, cat. # CC-0228) or from reduction mammoplasty tissue supplied by Dr. Ron Tickman. Breast epithelial cells were isolated according to published protocols (2, 4, 36). Briefly, the samples were minced and enzymatically digested to isolate mammary glands and single cells. After an initial plating in serum-containing medium, cells were cultured in serum-free mammary epithelial growth medium (MEGM) from Clonetics (cat. #CC-3051) or in DFCI-1 medium (1). Dr. Vito Quaranta, (The Scripps Research Institute, La Jolla) generously provided the breast carcinoma cell line, MDA-MB-435.

4. Flow Cytometry Analysis

Flow cytometry analysis was used to identify the integrin receptors expressed by cultured normal and transformed BC lines. Cells were incubated with anti-integrin Mabs (10 μg/ml or culture supernatant) for 30 min in suspension in FACS buffer (HBSS supplemented with 1% goat serum and 0.02% sodium azide). Cells were then washed and incubated with affinity purified FITC-conjugated goat anti-mouse, 2 mg/ml (Southern Biotechnology, Birmingham, AL). The appropriate isotype matched controls were included with each sample. Cells were analyzed by forward light scatter (linear) versus green fluorescence (log). Flow cytometric analysis of stained cells were performed on either a Coulter EPICS C System or a Becton Dickinson FACScan equipped with an argon laser. At least 5,000 events were analyzed for each anti-integrin antibody and compared to a matched isotype control.

5. Integrin clustering

Cells were first synchronized by culturing in the absence of any serum or exogenously added growth factors for 36 to 48 h. Monoclonal antibodies to specific integrin subunits were bound to the cells for 30 minutes at 4°C in growth factor-free media. Unbound primary antibodies were washed from the cells. The bound monoclonals were then cross-linked with the appropriate polyclonal secondary (rabbit anti-mouse or rabbit anti-rat) in media containing reduced levels of growth factors (1/10 to 1/100 of the normal media) and the cells were placed back at 37° C. Maximal clustering occurred approximately 30 minutes after addition of the cross-linking secondary antibodies and could often be detected within 15 minutes by immunofluoresence. Cells were lysed by scraping the cells into lysis buffer (1% NP-40 in 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin).

6. SDS-PAGE/ Western blots.

The protein concentration of each lysate was determined using the BCA protein assay kit (Pierce, Rockford, IL). Fifty or 100 micrograms of total protein were precipitated (41), resuspended in reducing sample buffer, and separated by SDS-PAGE. For immunoprecipitations, monoclonal antibodies were bound to goat anti-mouse Sepharose (Zymed, South San Francisco, CA) and incubated with 100 μg of total cell lysate overnight at 4°C. Immunoprecipitations were washed extensively with lysis buffer, eluted in reducing sample buffer, and separated by SDS-PAGE. Proteins were then transferred to PVDF membranes (Biorad, Hercules, CA) and probed with antibodies to the molecule of interest (retinoblastoma protein, cyclins, growth factor receptors). Bound antibodies were detected using either the colorimetric system of alkaline phosphatase conjugated to secondary antibodies along with NBT and BCIP as substrates (Promega, Madison, WI) or the ECL system of peroxidase conjugated secondary antibodies along with the ECL+Plus Western blotting detection system (Amersham, Arlington Heights, IL).

7. 96 well antibody-binding cell proliferation assays.

96 well microtiter plates, which were pre-coated with goat anti-mouse antibodies, were obtained from Pierce (Rockford IL). Plates were blocked with 0.5% heat-denatured bovine serum albumin (BSA) in phosphate buffered saline (PBS). Monoclonal antibodies diluted in blocking buffer were added at 2 μg/well in triplicate and incubated overnight at 4° C. Wells were washed three times with PBS and 3000 cells were added per well in media containing 1/100 dilution of serum or growth factors. Prior to addition to the wells, the cells were synchronized by serum deprivation for either 24 h (MDA-MB-435) or 48 h (MCF12A). The time of synchronization was based on growth curves under varying serum concentrations. Cultures were allowed to grow for 2-5 days at which time cell growth was assessed using the Cell Titer 96® Aqueous One Solution Reagent (Promega, Madison WI). This assay is based upon the ability of viable cells to reduce the tetrazolium compound [3-94,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (3, 10). Absorbance of each well was read at 490 nm in a plate reader.

8. Integrin α6 RT-PCR

Cells (HMEC, MCF12A, MDA-MB-435) were grown to near confluence. Frozen tissue sections of normal breast, in situ/invasive carcinoma, invasive carcinoma, and lymph node metastasis were obtained from the Department of Pathology, University of Michigan. Tissue sections were scraped into 1.5 ml eppendorf tubes. Total RNA was isolated from either cultured cells or frozen tissue sections by solubilization directly into TRIZOL ® Reagent (Life Technologies, Gaithersburg, MD) as described by manufacturer's protocol. First strand cDNA was synthesized using the SuperSCRIPT™ Preamplification Kit (Life Technologies, Gaithersburg, MD). PCR reactions for the integrin α6 alternatively spliced isoforms utilized nested oligonucleotides

(1156/1157-outer pair; 1681/2002-inner pair) as described previously(35). PCR products were resolved on TBE-agarose gels and visualized by ethidium bromide staining. Representative pcr products were subcloned using the TA Cloning® Kit (Invitrogen, San Diego, CA) and sequenced on an ABI Sequencer to verify that the products corresponded to either α6A or α6B.

B. Results and Discussion

Last year we reported that multiple integrins were capable of regulating cell cycle progression in normal human mammary epithelial cells (HMEC). That is, cell proliferation was stimulated in HMEC cultures synchronized by growth factor deprivation when cells were plated on ECM proteins (collagen I or laminin5) or when integrins were clustered using monoclonal antibodies cross-linked by polyclonal antibodies.

During this past year, we expanded our studies to the normal mammary epithelial cell line MCF12A and to the metastatic breast carcinoma cell line MDA-MB-435. Cells synchronized by serum deprivation were plated onto wells coated with monoclonal antibodies to integrins in media containing 0.1% fetal bovine serum. The number of cells present in each well was assayed 2-5 days after plating. MCF12A cells were stimulated to proliferate by antibodies to integrin subunits β1, α3, β4, α6, and αV (Fig 1).

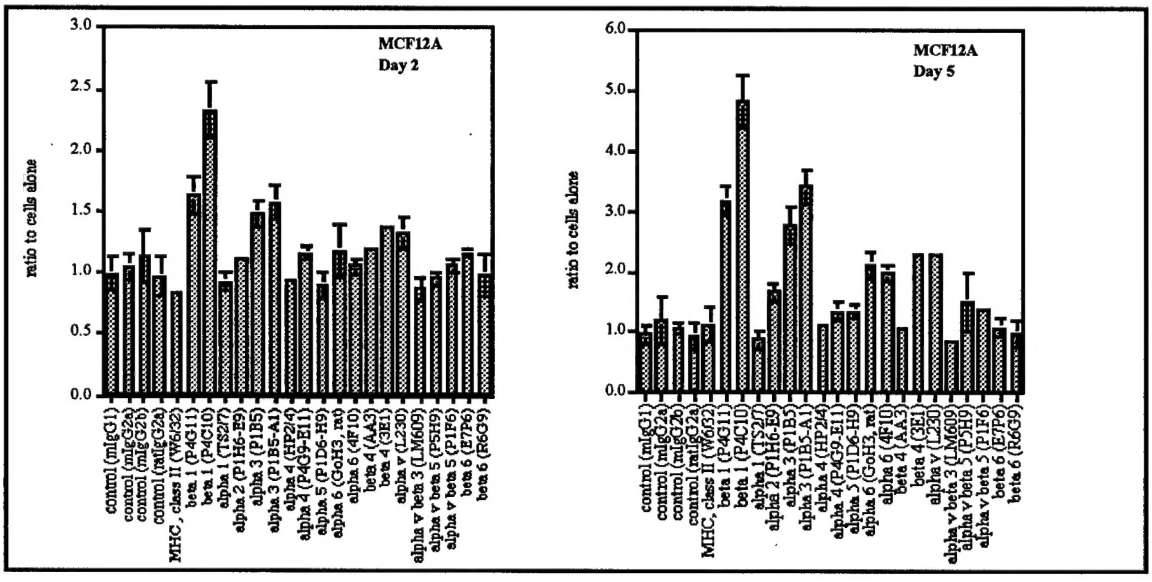

Fig. 1. Stimulation of MCF12A cell proliferation by anti-integrin antibodies in 0.1% FBS. Synchronized cultures of MCF12A cells were plated onto 96 well microtiter plates coated with anti-integrin antibodies in medium containing 0.1% FBS. Cell proliferation was assayed 2 and 5 days later as described in Methods. The data are expressed as a ratio of O.D. 490 nm values relative to cells plated on wells containing just the capturing goat anti-mouse antibodies.

Antigen (MAb clone)	MCF12A (MFI)	cell proliferation ratio, day5
control (anti-mIgG-FITC)	7.9	1.0 (cells alone)
control (mIgG1)	7.8	0.94
control (mIgG2a)	7.7	1.18
control (mIgG2b)	7.7	1.05
control (anti-ratIgG-FITC)	8.2	ND
control (rIgG)	63.8	0.93
β1 (P4C10-D4)	158.9	4.82
α2 (P1H6-E9)	39.3	1.65
α3 (P1B5)	173.9	2.75 and 3.42
α4 (P4G9-E11)	16	1.33
α5 (P1D6)	12.3	1.34
α 6 (GoH3, rat)	520.4	2.12
β 4 (3E1)	212.9	2.29
αV (L230)	42.8	2.28
αVβ 3 (LM609)	7.9	0.82
αVβ5 (P5H9-E3)	19.8	1.49

TABLE I Comparison of MCF12A FACS and cell proliferation data. The mean fluorescence index (MFI) obtained by FACS analysis of an asynchronous culture of MCF12A cells is compared with cell proliferation ratio plotted in Fig 1.

Antibodies to integrin subunits α2, α4, α5, and integrin αvβ5 had minimal if any effect. Antibodies to MHC class I complex (W6/32) did not stimulate cell proliferation. These results may reflect differences among integrins to regulate cell proliferation or may reflect the relative level of integrin expression on the cell surface (Table I). That is, there may be a "threshold" along the integrin signaling pathways that must be reached before the signal to initiate cell proliferation is transmitted. We have not yet determined whether or not serum deprivation alters integrin expression .

By contrast, plating MDA-MB-435 cells onto anti-integrin antibody coated wells produced effect either 2 days or 4 days after plating (Fig 2). These results suggest that there may be defects in the integrin regulation of cell proliferation in some breast carcinomas. Alternatively, the assay may need to be optimized for MDA-MB-435 cells. A reduction of media components by 50% resulted in a greatly reduced stimulation of cell proliferation in MCF12A cells (data not shown). Studies are underway to look at the effects of media conditions on this assay. Since MDA-MB-435 cells are highly metastatic, it is also possible that they produce levels of extracellular proteases that degrade the anti-integrin antibodies to disrupt the assay. However, extracellular

collagenase activity (zymogram gels) was barely detected in the media of cells cultured in 0.1% FBS (data not shown).

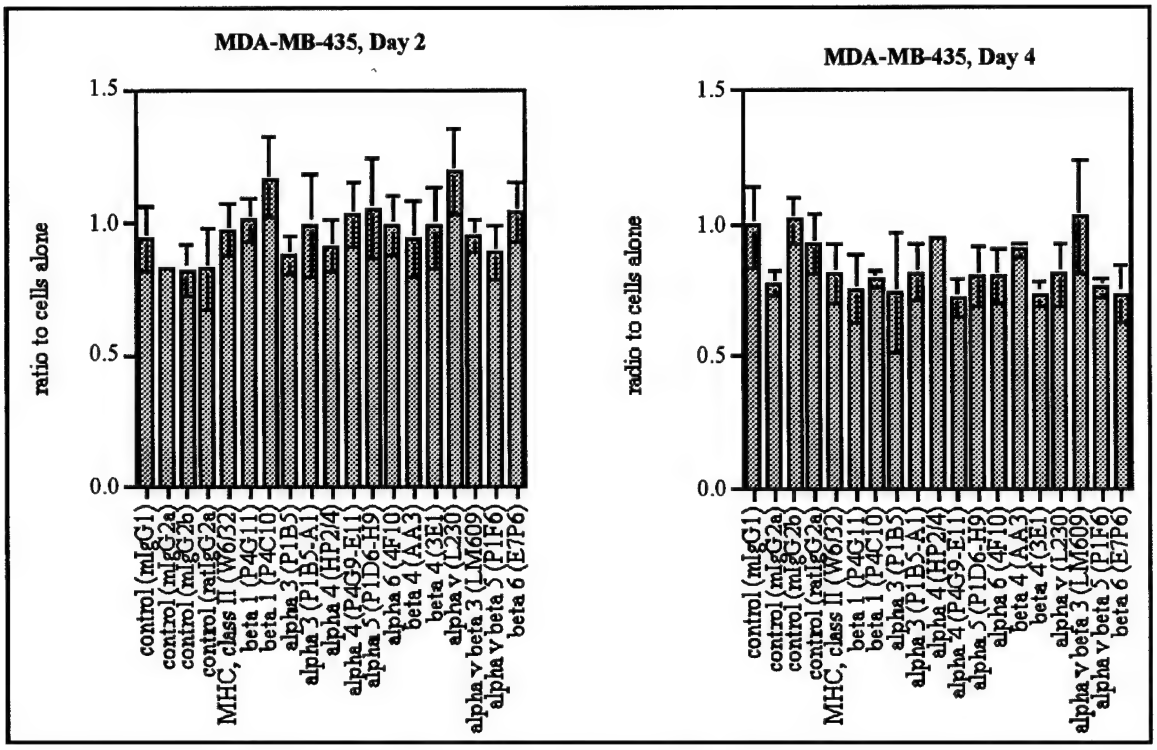

Fig. 2. Cell proliferation of MDA-MB-435 carcinoma cells is not stimulated by anti-integrin antibodies in 0.1% FBS. Synchronized cultures of MDA-MB-435 cells were plated onto 96 well microtiter plates coated with anti-integrin antibodies in medium containing 0.1% FBS. Cell proliferation was assayed 2 and 5 days later as described in Methods. The data are expressed as a ratio of O.D. 490 nm values relative to cells plated on wells containing just the capturing goat anti-mouse antibodies.

One possible mechanism of integrin regulation of cell proliferation is through the association and activation of growth factor receptors with activated integrin clusters as suggested by Plopper et al (28) and Miyamoto et al. (26). To date, we have not observed an association of growth factor receptors with integrins. Western blot analyses of integrins immunoprecipitated from MCF12A whole cell lysates did not detect an association with EGF receptor (EGFR) although EGFR was readily detected in the whole cell lysate (Fig 2A). The level of EGFR in MCF12A cells is much higher than in normal HMEC. Fig 2B shows that equivalent amounts of protein were analyzed in the MCF12A and HMEC cell lysates.

An initial experiment suggested that the EGF-like growth factor receptor erbB2 may be associated with β1 integrins in MCF12A cells. However, we have been unable to demonstrate this unequivocally and we have also been unable to detect erbB2 in whole

cell lysates of either MCF12 A or HMEC cells. We also have not detected FGFR or PDGFR in co-immunoprecipitation experiments with integrins. Although the integrin α

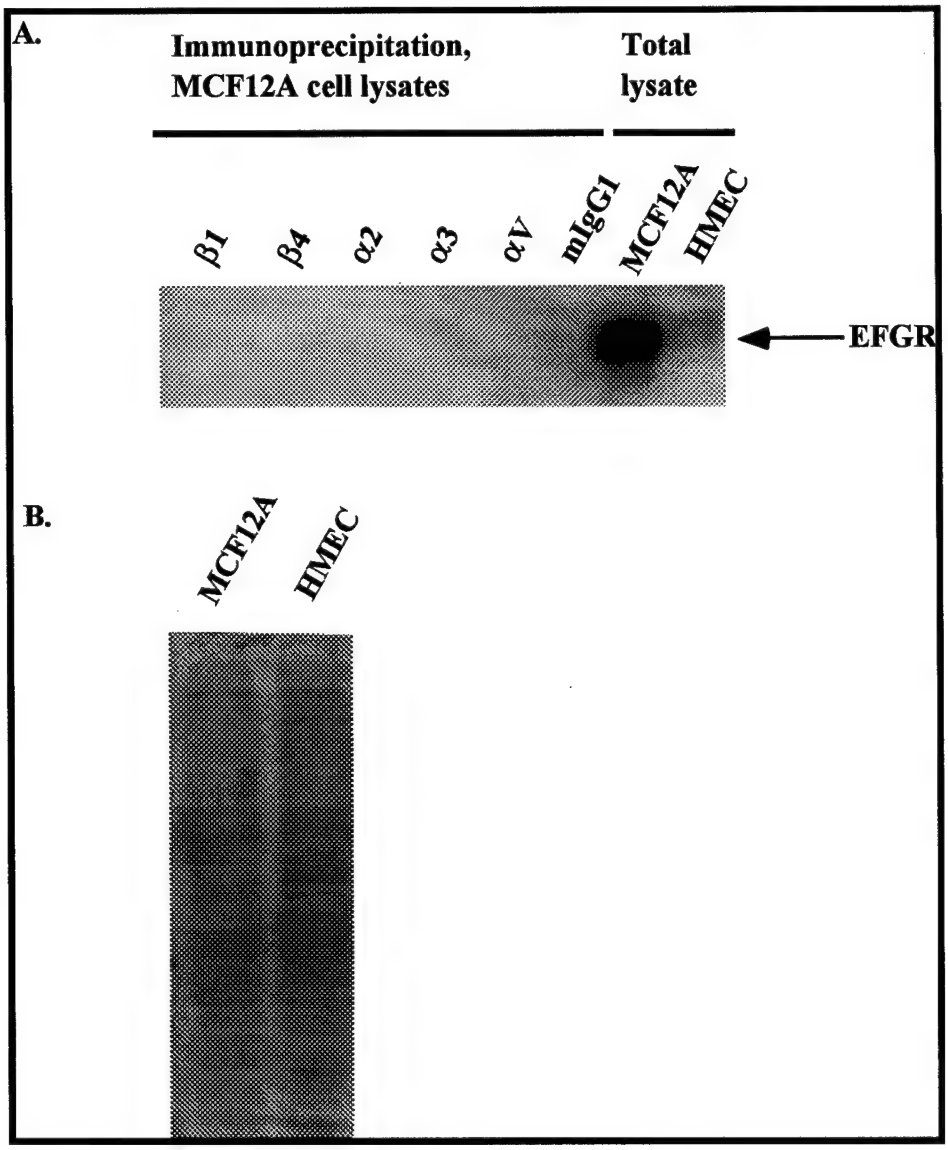

Fig. 2 EGFR does not appear to be associated with integrins, but is expressed at much higher levels in MCF12A cells than in HMEC. A) Western blot for EGFR in integrin immunoprecipitations of MCF12A cells or total cell lysates of MCF12A or HMEC. B).Coomasie-blue staining of total cell lysate lanes of blot membrane used for Western blot in A.

and β subunits remain associated during immunoprecipitation, it is possible that our lysis or immunoprecipitation buffers are too harsh and are disrupting weaker protein-protein binding interactions. To address this possible problem with the co-immunoprecipitation experiments, we attempted to look for co-localization of growth factor receptors with integrin clusters by immunofluoresence of acetone-fixed cells. Integrin clusters were

readily detected between 15 to 30 minutes after cross-linking with polyclonal secondary antibodies. Unfortunately, we have been unable to obtain a clear understanding of what is happening to the distribution of growth factor receptors (EGFR, erbB2, FGFR, or PDGFR) during the formation of these integrin clusters.

Cell synchronization by the removal of growth factors or serum blocks cell growth in the G1 phase of the cell cycle. Although cyclin D1 has been reported to be quite labile upon removal of growth factors (22-24), growth factor deprivation for 26 h did not eliminate cyclin Ds completely from HMEC cultures (Fig 3, T0 lanes). The appearance of a third band migrating slightly faster than the other two bands in the last 3 lanes of this blot suggests that cyclin D3 expression is induced by the re-addition of growth factors (Fig 3, T16, GF+ lanes). Clustering of β1 integrins at the time release did not appear to have a significant impact on the levels of cyclin D(s).

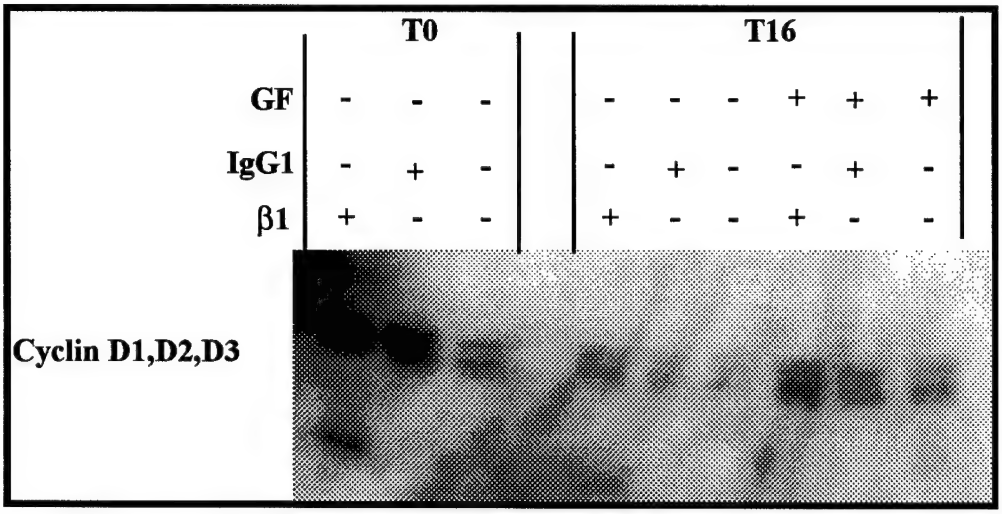

Fig. 3. Clustering of b1 integrins does not appear to alter the expression of cyclin D(s). Beta 1 integrins on HMEC cells were clustered using monoclonal antibodies in the absence (-) or presence (+) of growth factors. IgG1 samples are cells incubated with nonspecific IgG1 instead of the anti-b1 monoclonal antibody, P4C10. Twenty five micrograms of total cell lysate protein was separated by SDS-PAGE, transferred to Immobilon membrane, and probed with monoclonal antibodies which recognize cyclins D1, D2, and D3. The lower molecular weight band present in the T0, β1 (+) lane is the light chain from P4C10 which is recognized by the secondary antibody-enzyme conjugate used in the Western blot procedure.

We need to utilize monoclonal antibodies specific to cyclin D1, D2, or D3 which are not cross reactive to determine which cyclin Ds are being observed in this experiment (see Fig 5). Also, since Rb phosphorylation began around 8 hours after integrin clustering (1997 report, Fig 3), these studies should be repeated with time points between 0 and 8 hours after integrin clustering. We have also looked at the time course of cyclin A expression upon release from the serum-deprivation block. Cyclin A proteins were first detected 16 h after release of MCF12A cells into media containing 0.1% FBS or 1.0% FBS and

increased in amount up to 24 h (data not shown). However, we do not know if integrin clustering alters this time course of expression.

Analyses of detergent lysates of asynchronous cultures of HMEC, MCF12A, and MDA-MB-435 cells revealed that cyclin D2 was expressed by normal HMEC cells but not by either MCF12A or MDA-MB-435 cell lines. Initial comparison between MCF12A and normal HMEC cells with monoclonal antibodies cross-reactive with cyclins D1, D2, and D3 suggested that MCF12A expressed cyclins D1 and D3, but not D2 and that normal HMEC cells expressed cyclins D1 and D2, but little or none of cyclin D3 (Fig 4). Two different cultures of normal HMEC were analyzed: HMEC96.1 was isolated in this laboratory from a breast reduction specimen and HMEC 4144 was purchased from Clonetics (San Diego, CA). Two different cultures of MCF12A cells were also analyzed: cells cultured in the presence of 10% FBS (F/DV-C) or cells adapted to growth in the HMEC-defined growth medium, DFCI-1.

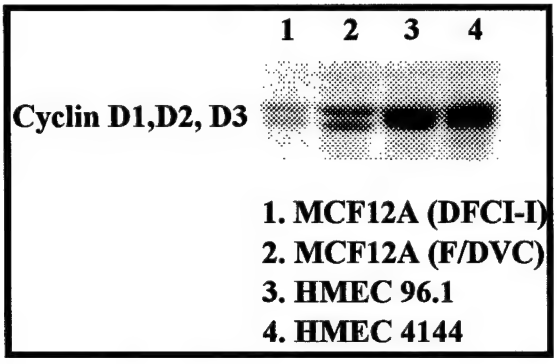

Fig. 4. Expression of cyclins D1, D2 and D3 in MCF12A and normal HMEC cells. One hundred micrograms of total cell lysate protein from asynchronous cultures were separated by SDS-PAGE, transferred to Immobilon membrane, and probed with monoclonal antibodies cross-reactive with cyclins D1, D2, and D3. The migration of bands suggest that MCF12A express cyclins D1 and D3 but not D2 while HMEC express cyclins D1 and D2, but not D3.

These results were confirmed using monoclonal antibodies specific for cyclin D1, cyclin D2, or cyclin D3. Fig 5 demonstrates that cyclin D1 and cyclin D3 are expressed by HMEC, MCF12A, and MDA-MB-435 cells while cyclin D2 is expressed only by HMEC cells. The predominant cell type in the normal HMEC cultures correspond to basal mammary epithelial cells. However, there are luminal cells present and these cultures have a limited life span before undergoing terminal differentiation. Studies are currently underway to determine if the expression of cyclin D2 is restricted to a particular cell population within the HMEC cultures.

Numerous attempts were made to determine if integrin clustering stimulated Rb phosphorylation in MCF12A and MDA-MB-435 cells. Unfortunately, we encountered a myriad of technical problems that have prevented us from determining the impact of integrin signaling on G1 cell cycle progression in these cell lines. Some of these problems

involved insufficient resolution of phosphorylated forms of Rb possibly due to the use of different protein precipitation techniques (acetone or trichloroacetic acid instead of our standard use of chloroform/methanol), use of an incorrect acrylamide percentage during SDS-PAGE, and high backgrounds during the Western blot procedure. There are also a number of parameters, such as the length of time for cell synchronization and the concentration of serum or growth factors, which may require optimization for each cell type or cell line. For example, Fig 3 (T16, Gf (-) lanes) demonstrates that levels of cyclin D1 and cyclin D2 decreased further when HMEC cultures were maintained for an additional 16 h in growth factor-free media. Also, the addition of growth factors/serum at 1/100 dilution of the standard media concentration is sufficient to stimulate some cell growth in cells synchronized in growth factor/serum-free media.

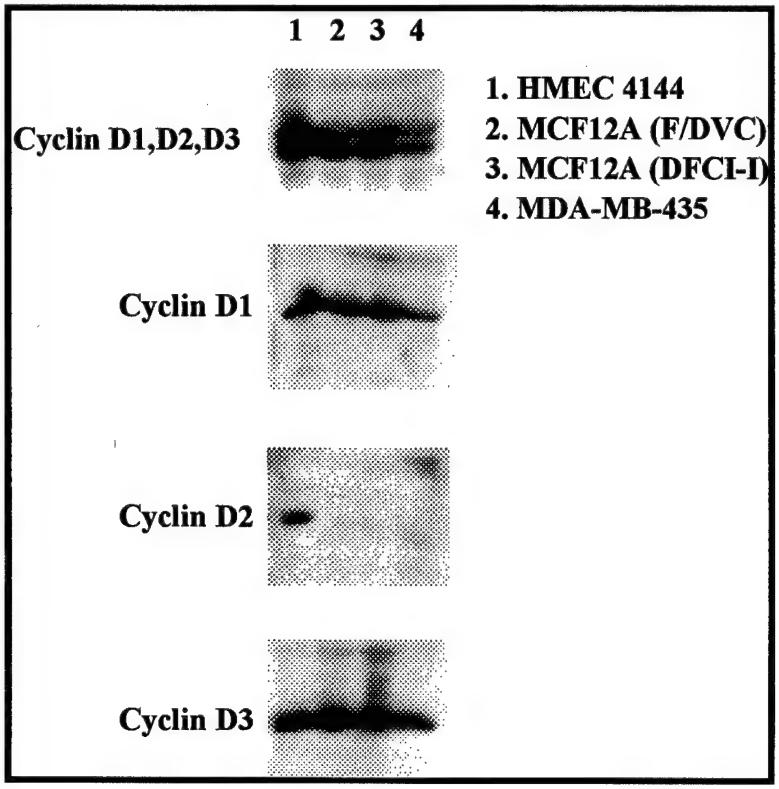

Fig. 5. Cyclin D2 is expressed by HMEC cells but not by MCF12A or MDA-MB-435. Western blot analyses of 50 mg of total cell lysate protein from asynchronous cultures of HMEC, MCF12A, or MDA-MB-435 cells for the expression of cyclins D1, D2, and D3. Monoclonal antibodies were either cross-reactive with all three cyclin D(s) or were specific to only one cyclin D.

The integrin α6 exists as two alternatively spliced forms (α6A and α6B) that differ in their cytoplasmic domains (9, 14, 35). In general, the α6A isoform is expressed by normal epithelial cells, such as epidermal keratinocytes while α6B is expressed by most carcinomas and tends to be the predominant form expressed by teratocarcinoma cell lines and totipotent or pluripotent cells such as mouse embryonic stem cells. The expression

of α6B may have a direct role in epidermal tumorigenesis (37). We were therefore interested in characterizing the pattern of α6 isoform expression in normal HMEC and mammary carcinomas. Analyses by RT-PCR revealed that normal HMEC and the normal mammary epithelial cell line MCF12A express only the α6A isoform while the highly metastatic mammary carcinoma cell line MDA-MB-435 expresses both α6A and α6B (Fig 6).

In order to determine if these observations are relevant in vivo, we attempted to isolate RNA from frozen sections of normal breast, in situ/invasive ductal carcinoma, invasive

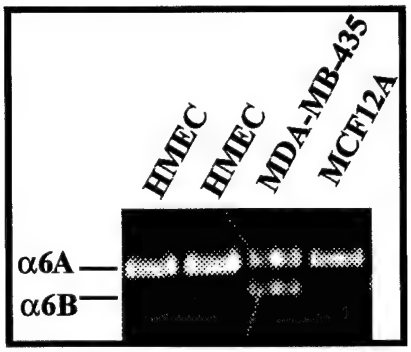

Fig. 6. The integrin α6B isoform is expressed by MDA-MB-435 cells but not by HMEC nor MCF12A cells. Total RNA was isolated from HMEC, MDA-MB-435, or MCF12 and analyzed for α6A and α6B expression by RT-PCR as described in Methods.

carcinoma, and a lymph node metastasis for α6 RT-PCR. The invasive carcinoma and the lymph node metastasis appear to express both isoforms of the integrin α6 (Fig 7). We have been unable to obtain reproducible PCR products from normal or in situ/invasive tissue sections. Hematoxylin and Eosin stained sections from these tissues show that the invasive carcinoma or lymph node metastasis samples have much higher cellular content than the normal or in situ/invasive samples which probably accounts for the differences in obtaining a6 PCR products. These results do not demonstrate that the breast cancer cells express both α6 isoforms. These assays have also been plagued by contamination in the negative control reactions which have produced an apparent α6B band in samples not reacted with reverse transcriptase and sometimes in the water only control (Fig 7, lanes 11, 13, and 14). The presence of an apparent α6B PCR product in the water control for the nested oligo pair suggests that either these oligos are contaminated with α6B or that the oligos are generating an artifactual product the same size as α6B. We are in the process of cloning and sequencing this PCR product to get a better understanding of how to correct this problem.

We will continue our studies of α6A and α6B expression during breast cancer progression. We will attempt to isolate RNA from blocks of tissue frozen in O.C.T.(17)

which would obviate the problem of amounts of starting material encountered when starting from tissue sections. If there appears to be a pattern of α6 isoform expression that correlates with the progression of breast cancer, we will attempt to identify the cell type(s) expressing each isoform by oligonucleotide hybridization of tissue sections (21).

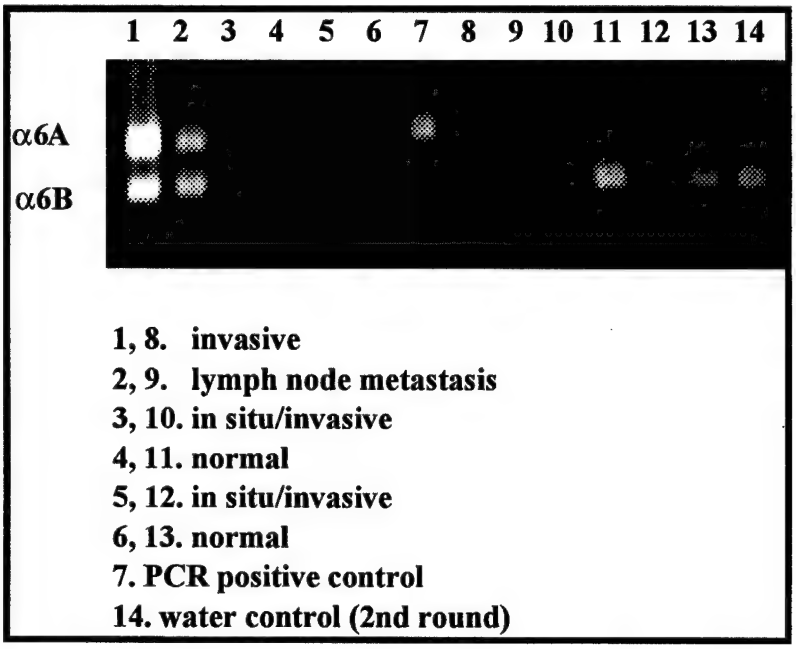

Fig 7. The α6B integrin isoform is expressed in invasive breast carcinoma and breast lymph node metastatic tissue. Total RNA was isolated from frozen tissue sections and analyzed for α6A and α6B by RT-PCR as described in Methods. Lanes 8-13 were RT-PCR reactions in which RT was omitted from the first strand cDNA synthesis reaction. Lane 14 is a water control using only the nested set of oligos (2nd round).

III. CONCLUSIONS

To date, the cell proliferation assay using antibody coated plates has provided the most consistent and compelling evidence for integrin-mediated regulation of cell proliferation. Initial results suggest that integrin regulation occurs in normal (MCF12A) but perhaps not in metastatic (MDA-MB-435) mammary epithelial cells. However, these results require a more extensive examination of assay conditions (e.g. serum concentrations, media compositions) and a more extensive examination of cell response (cell attachment, spreading, death) during the course of the assay. Differences in the strength of stimulation may reflect functional differences among the integrins or the level of integrin cell surface expression.

The mechanism(s) of integrin regulation of cell proliferation are still unclear. We have not observed a significant impact of integrin clustering on either the level or time course of

cyclin D(s) expression or Rb phosphorylation. We also have not observed an association of growth factor receptors (EGFR, c-erbB2, PDGF, FGFR) with integrins.

We will utilize the antibody cell proliferation assay to optimize our cell synchronization experiments for length of cell synchronization and growth factors/serum concentrations. We will then use these parameters to determine if integrin clustering alters the levels or time course of expression of various cell cycle components (cyclin D(s), cyclin A, cyclin E, and Rb phosphorylation). We will also expand our analyses to the expression of a number of cyclin dependent kinase inhibitors: p15, p16, p18, p19, p21, p27, and p57.

We have observed that the G1 cyclin, cyclin D2, is expressed in cultures of normal HMEC but not by either the normal mammary epithelial cell line, MCF12A, or the metastatic carcinoma cell line, MDA-MB-435. Several other human breast carcinoma cell lines (MCF-7, ZR-75, T-47D) and other breast cell lines (HBL-100, MCF-10A) have been reported to lack cyclin D2(31, 43). Buckley et al (5) also reported that the expression of cyclin D2 was lower in breast cancer cell lines than in cultures of normal breast epithelial cells. Thus, it appears that the establishment of a cell, normal or tumorigenic, may be correlated with a loss of cyclin D2 expression. The significance of this loss in expression is unclear. Cyclin D1, D2 and D3 are a family of related proteins involved in regulation of progression through the G1 phase of the cell cycle. However, several differences have been reported among the cyclin D(s) for their binding to specific cdk(s), the formation of active or inactive kinase complexes, and binding to Rb protein (31,11, 13, 20, 34). In the study of mouse mammary tumorigenesis, Said et al (29) suggested that cyclin D2 binding to cdk4 in hyperplasias may be an attempt to maintain or induce differentiation of the mammary epithelial cells. Further investigation into the functions of cyclin D2 in normal human mammary epithelial cells appears warranted. We will examine the expression of cyclin D2 and its associations with cdk2 and cdk4 during progression through the cell cycle.

Preliminary results also suggest that the expression of the integrin α6B isoform may correlate with tumorigenesis. We will continue our efforts to determine if such a correlation exists and examine how α6B may contribute to the progression of breast cancer.

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V. Appendix

A. Bibliography of publication and meeting abstracts.

Publications: None

Abstracts:

Tamura, R.N., Pabich, W., Symington, B., and Wayner E. (1997) Role of integrins in regulating cell proliferation of normal and neoplastic mammary epithelial cells. Era of Hope, The Department of Defense Breast Cancer Research Program Meeting, October 31, November 4, 1997.

B. List of Personnel:

1. Richard Tamura, Ph.D., Principal Investigator
2. Elizabeth Wayner, Ph.D., Principal Investigator
3. Wendy Pabich, Research Technician I

Abstract for: Era of Hope, The Department of Defense Breast Cancer Research Program Meeting, October 31 to November 4, 1997, Washington D.C.

ROLE OF INTEGRINS IN REGULATING CELL PROLIFERATION OF NORMAL AND NEOPLASTIC MAMMARY EPITHELIAL CELLS.

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The extracellular matrix (ECM) is a milieu of proteins which provide structural integrity to tissues and organs as well as cues regulating several cell functions such as proliferation, migration, differentiation, and gene expression. These cell-ECM interactions are mediated, in part through the integrin family of cell adhesion receptors.

We have been investigating the role of integrins in the regulation of cell cycle progression. Initial experiments suggested that peptides containing the cell adhesion motif of Arg-Gly-Asp (RGD) bound to the integrin $\alpha5\beta1$ and regulated cdc2 kinase/cyclin A associations in normal human epidermal keratinocytes and the non-tumorigenic breast cancer cell line HBL 100 but not in the tumorigenic cell line BT20 or virally-transformed keratinocytes. These results suggested a potential defect in the $\alpha5\beta1$ signaling pathway regulating cell cycle progression in transformed cells. We extended these studies to normal mammary epithelial cells (HMEC) and to a number of additional breast cancer cell lines (T47D, DU4475, MDA-MB-453, MDA-MB-157, BT 483, Hs578t, MDA-MB-361, MCF7, BT474, and MDA-MB-134-IV) which vary from being partially transformed (agar growth/non-tumorigenic) to fully transformed (tumorigenic/metastatic). In order to understand the interaction of the RGD peptides with these various cell lines and HMEC, we characterized the repertoire of integrin expression by FACS. Of the three integrins which bind soluble RGD peptides with the highest avidity, $\alpha v\beta5$ was expressed by nearly all of the cell lines, 40% expressed $\alpha v\beta3$, and 60% expressed $\alpha5\beta1$. HMEC expressed intermediate levels of $\alpha5$ and $\alpha4$, low levels of $\alpha6$ and $\alpha v\beta5$, and were negative for $\alpha v\beta3$. The most prominent integrin expressed by HMEC and by all of the cell lines was $\alpha3\beta1$, which is primarily a receptor for laminins but also mediates adhesion to fibronectin and collagens.

Incubation with RGD peptides resulted in increases in both cdc2 kinase activity and cyclin A associated kinase activity in HMEC and a number of the cell lines. However, in most cases, the stimulation of cdc2 kinase activity or cyclin A associated kinase activity appeared to be mutually exclusive suggesting a mechanism that is distinct from that of normal human epidermal keratinocytes or HBL100 cells. There was also no apparent

correlation between the stimulation of cell cycle kinases with the expression of the integrin α5β1, αvβ3, or αvβ5. Nor did there appear to be a correlation between the stimulation of cell cycle kinases with the tumorigenic potential of the cell line.

Immunohistochemical analysis of normal and tumor breast tissue indicated that _5_1 was not expressed by either normal mammary epithelial cells or tumor cells. Basal mammary epithelial cells expressed integrins α3, α6, β4, α2, β1, αvβ5, and possibly αvβ6, luminal cells and cancer cells (in situ and invasive ductal) expressed predominately α3β1. A few samples were positive for αvβ3 in the basal cells. The integrin α4β1 was not expressed by either normal epithelial cells or cancer cells. These results suggest that α5β1 is not of primary importance in mediating cell adhesive signaling events in either normal mammary epithelial cell or during tumorigenesis.

Recently, we have initiated studies to examine cell proliferation regulation mediated through specific integrins by clustering with monoclonal antibodies on cells synchronized to Go by serum/growth factor deprivation. Preliminary results suggest that a number of integrins, β1, αv, α3, and β4, may promote transit of normal HMEC through the cell cycle under reduced serum conditions as evaluated by the time course of retinoblastoma (Rb) phosphorylation. Similar results have been demonstrated by others for β1 integrins in fibroblast cells and is thought to involve promotion of growth factor receptor clustering and activation of mitogen activated protein kinase (MAPK). We are looking to see if similar mechanisms are being used by normal mammary epithelial cells, both basal and luminal, and breast cancer cells. Understanding the role of integrins in the regulation of cell proliferation may lead to the development of novel therapeutics for the treatment of breast cancer.